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Development of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) thermal inactivation method with preservation of diagnostic sensitivity

Young-Il Kim[1,2†], Mark Anthony B. Casel[1,2†], Se-Mi Kim[1], Seong-Gyu Kim[1], Su-Jin Park[1,2], Eun-Ha Kim[1,2], Hye Won Jeong[1], Haryoung Poo[3], and Young Ki Choi[1,2*]

[1]*College of Medicine and Medical Research Institute, Chungbuk National University, Cheongju 28644, Republic of Korea*
[2]*Zoonotic Infectious Diseases Research Center, Chungbuk National University, Cheongju 28644, Republic of Korea*
[3]*Infectious Disease Research Center, Korea Research Institute of Bioscience and Biotechnology, University of Science and Technology, Daejeon 34141, Republic of Korea*

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Various treatments and agents had been reported to inactivate RNA viruses. Of these, thermal inactivation is generally considered an effective and cheap method of sample preparation for downstream assays. The purpose of this study is to establish a safe inactivation method for SARS-CoV-2 without compromising the amount of amplifiable viral genome necessary for clinical diagnoses. In this study, we demonstrate the infectivity and genomic stability of SARS-CoV-2 by thermal inactivation at both 56°C and 65°C. The results substantiate that viable SARS-CoV-2 is readily inactivated when incubated at 56°C for 30 min or at 65°C for 10 min. qRT-PCR of specimens heat-inactivated at 56°C for 30 min or 65°C for 15 min revealed similar genomic RNA stability compared with non-heat inactivated specimens. Further, we demonstrate that 30 min of thermal inactivation at 56°C could inactivate viable viruses from clinical COVID-19 specimens without attenuating the qRT-PCR diagnostic sensitivity. Heat treatment of clinical specimens from COVID-19 patients at 56°C for 30 min or 65°C for 15 min could be a useful method for the inactivation of a highly contagious agent, SARS-CoV-2. Use of this method would reduce the potential for secondary infections in BSL2 conditions during diagnostic procedures. Importantly, infectious virus can be inactivated in clinical specimens without compromising the sensitivity of the diagnostic RT-PCR assay.

***Keywords*:** SARS-CoV-2, heat inactivation, COVID-19, genomic stability, RT-PCR

[†]These authors contributed equally to this work.
*For correspondence. E-mail: choiki55@chungbuk.ac.kr; Tel.: +82-43-261-3384; Fax: +82-43-273-3252
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Introduction

Corona Virus Disease 2019 (COVID-19) is an infectious disease caused by a novel coronavirus, Severe Acute Respiratory Syndrome 2 (SARS-CoV-2). SARS-CoV-2, temporarily named *2019 novel coronavirus* (2019-nCoV) by the World Health Organization on January 7, 2020, is a positive-sense single-stranded RNA virus belonging to genus Betacoronavirus, which has distinct genetic characteristics from SARS-CoV and MERS-CoV (Huang *et al.*, 2020; WHO, 2020b). Due to the highly contagious nature of SARS-CoV-2, COVID-19 was designated a Public Health Emergency of International Concern by the World Health Organization (WHO) on January 30, 2020, and officially declared a pandemic on March 11, 2020 (WHO, 2020a, 2020c). As of August 12, 2020, there have been 20,330,351 confirmed cases and 742,413 case fatalities due to respiratory failure or other complications associated with this infection (ECDC, 2020). Although most people infected with SARS-CoV-2 suffer from mild to moderate respiratory illness and recover without requiring special treatment, patients with chronic health problems and immunocompromised conditions are more likely to develop serious illness, including mortality (Chan *et al.*, 2020).

Coronaviruses are known to be fragile and are vulnerable to heat (Lelie *et al.*, 1987; Lamarre and Talbot, 1989; Schlegel *et al.*, 2001), but appear to be stable at 4°C (Lamarre and Talbot, 1989; Pratelli, 2008). Thermal inactivation of viruses is directly related to the chemical composition of these microbes and involves disruption of the sugar-phosphate backbone or denaturation of the viral protein particle (Knight, 1975; Siddell *et al.*, 1983; Nuanualsuwan and Cliver, 2003; Decrey *et al.*, 2016; McDonnell, 2017). For the inactivation of highly pathogenic viruses, the Pan American Health Organization recommends that serum samples or other organic fluids be incubated at 60°C for 60 min. Therefore, this procedure has been adopted by many laboratories for the handling of highly pathogenic virus specimens (Pratelli, 2008; PAHO, 2020a, 2020b). Since many coronaviruses are sensitive to thermal inactivation, the WHO recommended that SARS-CoV specimens be placed at 56°C for 15 to 45 min for viable virus inactivation (WHO, 2003), a recommendation that can be adapted to heat inactivate SARS-CoV-2. In addition, other studies have suggested incubation at 65°C for 10 min is required for full inactivation of coronaviruses (Pratelli, 2008; PAHO, 2020a). Although 56°C and 65°C are commonly used temperatures for inactivation of enveloped viruses (Roehrig *et al.*, 2008; Cutts *et al.*, 2016; Park *et al.*, 2016), the difference in recommended temperature requirements

Table 1. Primer set specific for SARS-CoV-2 ORF1a, Spike, E gene, and N gene

	Forward sequence (5′ – 3′)	Reverse sequence (5′ – 3′)
ORF1a	CCC TGT GGG TTT TAC ACT TAA	ACG ATT GTG CAT CAG CTG A
S	ATT CAA GAC TCA CTT TCT TCC ACA	TGT TTA AAG CTT GTG CAT TTT GGT TGA CC
E	GTT GAT GAG CCT GAA GAA CAT GTC C	CGT ACC TGT CTC TTC CGA AAC G
N	GTC AAG CCT CTT CTC GTT CC	GAA GCG CTG GGG GCA AAT TG

is dependent on the type of sample and type of analysis to be completed after inactivation. However, the RNA stability in SARS-CoV-2 specimens after this heat inactivation has not been investigated in detail.

Given the rapid, worldwide geographic spread of COVID-19, rapid analysis of large numbers of clinical specimens for SARS-CoV-2 diagnosis could increase the chances of nosocomial transmission in diagnostic laboratories. Therefore, methods for the safe inactivation of SARS-CoV-2 in clinical specimens are essential to reduce any potential secondary transmission during diagnostic procedures; however, care must be taken not to affect diagnostic sensitivity. In this study, we establish thermal inactivation procedures for SARS-CoV-2 at two different temperatures (56°C and 65°C) and compare the physical stability of the RNA genome by amplifying the ORF1a, Spike, N, and E genes of heat-inactivated samples and comparing with non-treated clinical specimens.

Materials and Methods

Viruses and cell line

As a reference strain, we adapted a SARS-CoV-2 human isolate, CBNU-nCoV01 (S lineage, GISAID accession number: EPI_ISL_507039), which was isolated from a COVID-19 patient at Chungbuk National University, and the virus was grown in Vero cells (Cat# ATCC CCL-81) as previously described (Kim *et al.*, 2020). Briefly, Vero cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich) and supplemented with 10% fetal bovine serum (FBS) containing antibiotics (Gibco). When approximately 75% of the cell monolayer of a virus-infected flask showed the cytopathic effect (CPE), culture supernatant was harvested and stored at -80°C until thawed for use followed by centrifugation at 12,000 rpm for 10 min at 4°C to remove cell debris.

Determination of 50% tissue culture infectious dose ($TCID_{50}$)

Confluent Vero cell monolayers were grown on 96-well microtiter plates were infected with 50 μl of 10-fold serial dilutions of the stock virus in DMEM starting from 10^{-1} to 10^{-8}. Infected cells were incubated at 37°C with 5% CO_2 for 4 days. The appearance of CPE was recorded daily and the $TCID_{50}$ was determined according to the method of Reed and Muench (Reed and Muench, 1938). Titrations were done in replicates of three. Following the WHO recommendations, all work involving infectious SARS-CoV-2 was performed under biosafety level (BSL)-3 conditions in a BSL-3 facility.

Physical inactivation of SARS-CoV-2 and infectivity determination

Virus infectivity was assessed by performing virus titrations after heat inactivation treatments. To assess the efficiency of thermal inactivation, 0.5 ml supernatant from cell culture was used for the inactivation assay. The virus culture stock with a titer of $10^{5.5}$ $TCID_{50}$/ml was subjected to two temperatures (56°C and 65°C) using a heating block (DAIHAN Heating-Block, Maxtable H10) over different time periods (10 min, 15 min, 30 min, 60 min, and 120 min). The temperature in the tubes was monitored with a thermometer placed in the same amount of DMEM in a separate tube. Virus specimens from each treatment condition were used to infect a monolayer of Vero cells to evaluate the viral titer by $TCID_{50}$. All experiments were repeated at least three times under the same conditions.

Validation of nucleic acid stability after heat inactivation

To test whether the heat inactivation would degrade SARS-CoV-2 viral RNA, total RNA was extracted from 0.2 ml of each thermally inactivated SARS-CoV-2 sample using Qiagen Viral RNA Mini Kit (Qiagen) according to manufacturer's guideline. The viral RNA titers were quantified by Real-Time qRT-PCR as previously described (Kim *et al.*, 2020) using primer sets specific (Table 1) for ORF1a, Spike, N, and E-gene (Corman *et al.*, 2020; Li *et al.*, 2020) and the SYBR Green kit (iQTM SYBR Green supermix kit, Bio-Rad). The viral RNA copy number was calculated as previously described (Kim *et al.*, 2020).

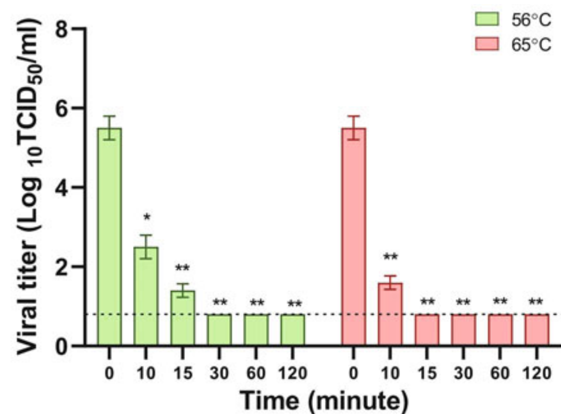

Fig. 1. Viral titer in cells of SARS-CoV-2 according to heat inactivated conditions. The mean viral titers ($TCID_{50}$/ml) obtained after inactivation of cell culture supernatants with $10^{5.5}$ $TCID_{50}$/ml of SARS-CoV-2 at time zero. The titers shown are means ± SD from three independently performed thermal inactivation at 56°C and 65°C on different time points (10 min, 15 min, 30 min, 60 min, and 120 min) and non-detection corresponds to titers below the limit of detection (0.8 log_{10} $TCID_{50}$/ml) (dashed lines). Green bars indicate viral titers from samples inactivated at 56°C and the red bars indicates viral titers from samples inactivated at 65°C. (* $p < 0.001$ and ** $p < 0.0001$)

Heat inactivation and measurement of viral titers in clinical COVID-19 specimens

Clinical COVID-19 specimens (n = 40) were collected from the National Medical Center and Chungbuk National University hospital from March to May 25, 2020. Furthermore, negative samples (n = 10) and unknown samples (n = 16) were added for sensitivity and specificity evaluation of the procedure. Each specimen was divided into two separate tubes; one was stored at 4°C (the no heat inactivation control), and the other one was incubated at 56°C in a thermal block for 30 min. Following heat inactivation procedure, specimens were subjected to qRT-PCR to compare cycle thresholds among treatments.

Statistical analysis

Data are presented as mean ± standard deviation (SD). Unpaired two-tailed t-tests were used to analyze the differences between paired samples in inactivated and non-inactivated samples. P-values less than 0.05 were considered statistically significant.

Results

Effect of thermal inactivation on viral infectivity

To clarify the temperature sensitivity of SARS-CoV-2, we evaluated the viable viral titers in Vero cells following treatment of $10^{5.5}$ $TCID_{50}$/ml SARS-CoV-2 with two different temperatures.

As shown in Fig. 1, heat treatment of SARS-Cov-2 at 56°C or 65°C for 30 min resulted in a rapid reduction of the viral infectivity titer below the detection limit. In detail, the CBNU-nCoV01 showed an observable decrease, from its original viral titer of $10^{5.5}$ $TCID_{50}$/ml to $10^{2.5}$ $TCID_{50}$/ml, in infectivity with as little as 10 min of heat inactivation at 56°C, and the viability of the virus was maintained even after 15 min of heat exposure although the viral titer was as low as $10^{1.5}$ $TCID_{50}$/ml. However, no viable virus was detected in the specimens treated for 30 min. At 65°C, the initial viral titer of $10^{5.5}$ $TCID_{50}$/ml dropped to $10^{1.5}$ $TCID_{50}$/ml after 10 min of heat incubation and viable CBNU-nCoV01 was not detected after 15 min of heat inactivation. This result demonstrates that SARS-CoV-2 is fully inactivated when incubated at 56°C or 65°C for 30 min or 15 min, respectively. A rapid decline in viral infectivity of SARS-CoV-2 was observed at the higher temper-

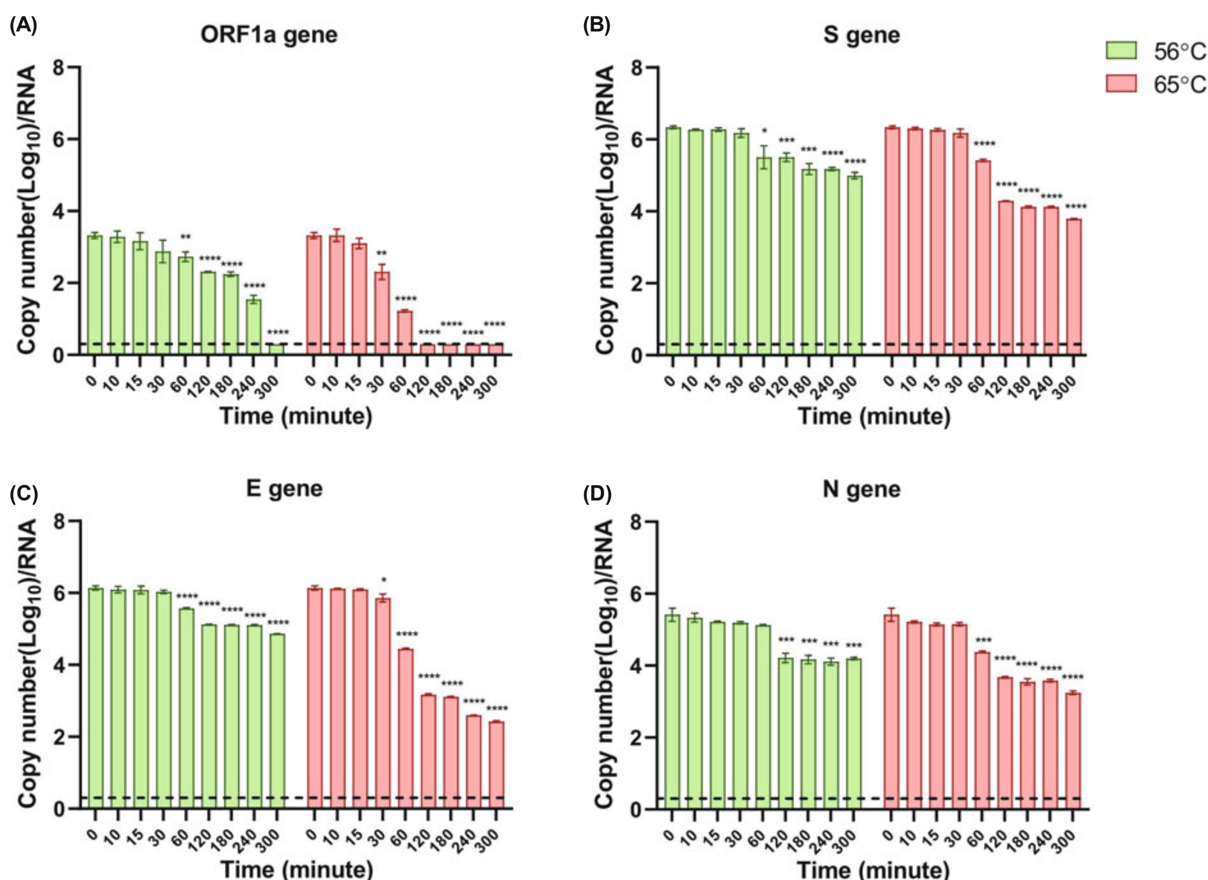

Fig. 2. **Quantitative PCR value of SARS-CoV-2 according to heat inactivated conditions.** Comparison of mean copy numbers (Log_{10})/RNA of ORF1a (A), Spike gene (B), E gene (C), and N gene (D) from samples inactivated at 56°C and 65°C on different time periods. Green bars indicate copy numbers from samples inactivated at 56°C and the red bars indicates copy numbers from samples inactivated at 65°C. The copy numbers shown are means ± SD from three independently performed experiments and titers below the limit of detection are shown as 0.3 log_{10} viral RNA copy numbers (dashed lines). ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$)

Table 2. Virus isolation on COVID-19 clinical specimens before and after heat inactivation (56°C for 30 min)

	Positive samples (n = 40)	Negative sample (n = 10)	Unknown sample (n = 16)
Number of isolated virus/ naïve samples	18/40	0/10	0/16
Number of isolated virus/ heat inactivated samples	0/40	0/10	0/16

ature (65°C), while a more gradual decrease in infectivity was seen with heat inactivation at 56°C.

Effect of heat inactivation on nucleic acid stability

To test whether heat inactivation could have a negative effect on viral RNA stability, $10^{5.5}$ $TCID_{50}$/ml of virus stock was subjected to either 56°C or 65°C in a thermal block. Following each step of heat inactivation, total RNA was extracted and qRT-PCR assays were performed to calculate the RNA copy numbers of ORF1a, S, E, and N gene segments (Fig. 2).

Heat inactivation at 56°C for up to 30 min did not cause a significant decrease in viral RNA copy numbers in comparison with the non-heat treated control for ORF1a (Fig. 2A, Green Bar), S (Fig. 2B, Green Bar), E (Fig. 2C, Green Bar), or N (Fig. 2D, Green Bar). In addition, for incubations within 30 min there was a mean decreased of 0.41 log_{10} copies/ml, 0.16 log_{10} copies/ml, 0.08 log_{10} copies/ml, and 0.26 log_{10} copies/ml RNA copy numbers were observed to ORF1a, S, E, and N, respectively, for every time period. The N gene showed comparable RNA copy numbers with that of the non-heat treated control specimens even after 60 min of heat inactivation, suggesting that this gene has the highest genetic stability at 56°C. However, there was a slight, but significant, decrease in RNA copy numbers with incubations longer than 30 min (Fig. 2A–D).

As for heat inactivation at 65°C (Fig. 2, Red Bar), there was no significant difference in viral RNA copy numbers during short incubations (10 to 15 min of thermal inactivation) between heat-treated and non-heat treated samples. In addition, for incubations within 15 min there was a mean decreased of 0.19 log_{10} copies/ml, 0.07 log_{10} copies/ml, 0.02 log_{10} copies/ml, and 0.31 log_{10} copies/ml were observed to ORF1a, S, E, and N, respectively. Meanwhile, there was a significant decrease in RNA detection when samples were inactivated for more than 15 min. Following 30 min of heat inactivation, decreases of approximately 2%, 4%, and 5% were seen for the RNA copy numbers of S, E, and N, respectively, and a significant drop of 15%, 27%, and 20%, for the S, E, and N genes occurred with 60 min of thermal inactivation. More-

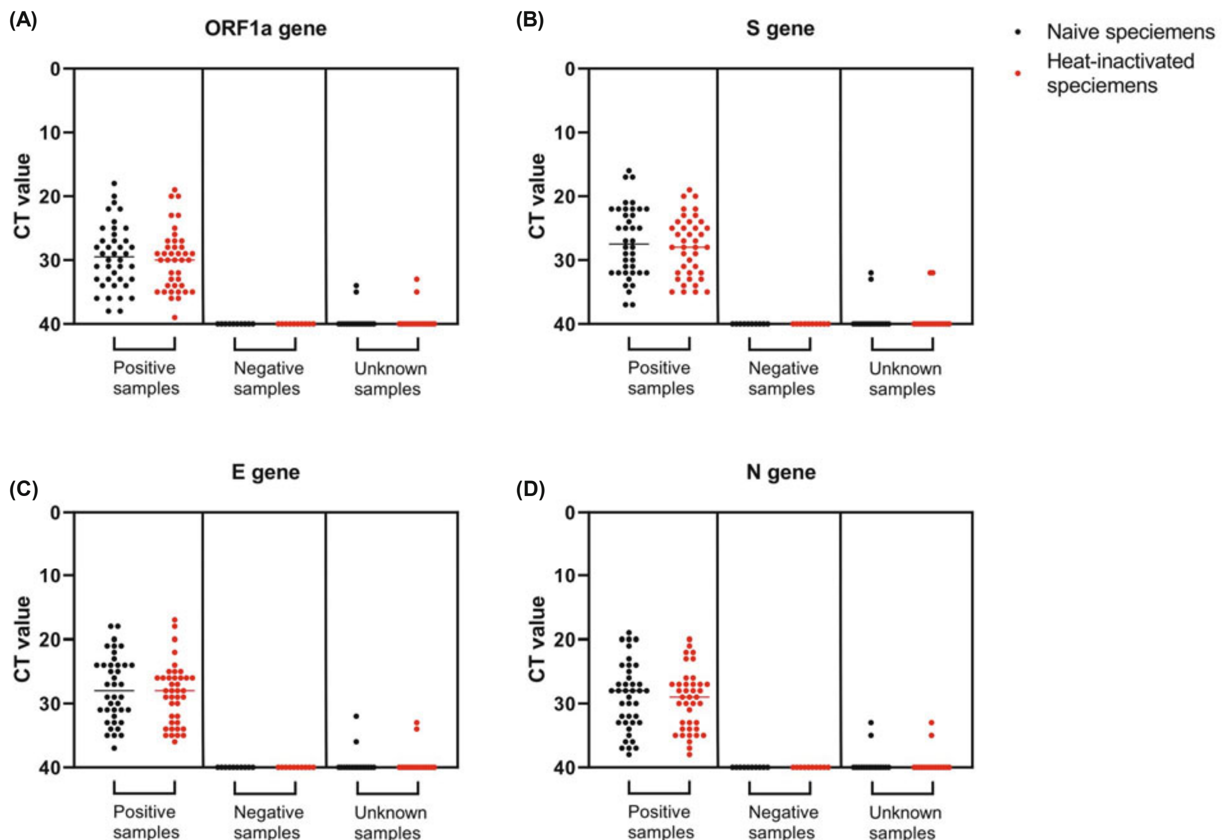

Fig. 3. The cycle threshold (Ct) value for ORF1a, S, N, and E gene of SARS-CoV-2 according to heat inactivation conditions in clinical samples. Comparison of cycle threshold (Ct) value of ORF1a (A), Spike gene (B), E gene (C), and N gene (D) from naïve (black) and heat inactivated specimens (red). Ct values are plotted as dots for each of the clinical specimens. The mean Ct value for each treatment is indicated by a horizontal bar.

over, at 120 min of heat inactivation the ORF1a gene RNA was significantly reduced below the quantification level. This data suggests that SARS-CoV-2 viral RNA remains stable when treated at 56°C or 65°C for up to 30 min.

Heat inactivation of clinical COVID-19 specimens

We next evaluated the viability and genomic stability of SARS-CoV-2 with laboratory-confirmed COVID-19 clinical specimens. We were able to isolate SARS-CoV-2 from 18 of 40 laboratory-confirmed nasopharyngeal specimens; however, no virus was isolated from specimens incubated at 56°C for 30 min (Table 2). Nonetheless, we were able to detect ORF1a, S, N, and E genes by qRT-PCR from these thermally inactivated specimens without significant differences in Ct values compared with non-inactivated specimens. Further, there was positive detection of target gene segments by qRT-PCR of two unknown samples with no significant difference between heat inactivated and naïve specimens. In addition, qRT-PCR on negative samples resulted in no amplification of the target gene segments in either inactivated or naïve specimens (Fig. 3). Thus, heat inactivation of COVID-19 clinical specimens at 56°C for 30 min successfully reduces the transmission risk, without compromising the ability to detect virus by qRT-PCR.

Discussion

Thermal inactivation of viruses occurs through denaturation of the secondary structures of viral proteins, thereby altering their conformation and function in attachment and replication within the host (Lelie *et al.*, 1987; Schlegel *et al.*, 2001). In addition, absorption of heat causes linked molecules to vibrate vigorously, leading to the disruption of bonding, such as in sugar-phosphate bonds, by creating nicks in the phosphodiester linkages and thus causing nucleic acid fragmentation (Knight, 1975; Siddell *et al.*, 1983; Nuanualsuwan and Cliver, 2003; McDonnell, 2017).

In this study, we found that the SARS-CoV-2 is efficiently inactivated following incubation at 56°C or 65°C for 30 min or 15 min, respectively. These results are consistent with reports of other studies on the effectiveness of subjecting biological specimens to heat in order to eliminate viral infectivity, including SARS-CoV, MERS-CoV, and human seasonal coronavirus strains (Herriott, 1961; Darnell *et al.*, 2004; Yunoki *et al.*, 2004; Leclercq *et al.*, 2014; Batéjat *et al.*, 2020). However, most of these studies were focused on virus inactivation for experimental purposes and not for use in diagnostic procedures. In this study, we demonstrate that the genomic stability of SARS-CoV-2 is maintained after 30 min of heat inactivation of specimens at 56°C, as evidenced by qRT-PCR. Further, we also verified the applicability of this heat inactivation method to clinical specimens from COVID-19 patients. It is noteworthy that heat inactivation of SARS-CoV-2 at 56°C for 30 min does not alter the sensitivity of RNA detection by qRT-PCR and similar results were obtained as with non-treated clinical specimens. Therefore, this result suggests that a simple pre-incubation of clinical specimens from COVID-19 patients in a thermal block can reduce the risk of laboratory acquired infections during the handling of clinical specimens and thus greatly increase testing ability.

Some intact positive-sense viral RNA genomes have been shown to act as an mRNA and thus, serve as a transcript resulting in the generation of viral proteins and genetic replication when introduced into cells under certain conditions (Larkin, 1977; Gamarnik and Andino, 1998). Thus, although we demonstrated high genetic stability of SARS-CoV-2 viral RNAs following thermal inactivation, the potential for infection by an intact, free RNA was not evaluated. Although the likelihood of such infection by intact SARS-CoV-2 RNA in diagnostic laboratory staff is very low, further cell-based studies are needed to clarify this concern.

Taken together, this study demonstrates effective inactivation of viable SARS-CoV-2 in experimental and clinical specimens by heat treatment. In addition, we also clearly demonstrate that inactivation of the virus at 56°C for 30 min improves the safety profile of clinical specimens while imparting no negative effect on the diagnostic assay for SARS-CoV-2 by qRT-PCR. Thus, this simple heat inactivation method can improve conditions for molecular diagnostic testing and mitigate the risk of exposure for laboratory personnel handling highly contagious virus specimens.

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Conflict of Interest

The authors have declared that no conflict of interest exists.

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